PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification [4] :		(11) International Publication Number: WO 89/07445
A61K 35/14, 37/02 // C12N 5/00	A1	(43) International Publication Date: 24 August 1989 (24.08.89)

(21) International Application Number: PCT/US89/00552

(22) International Filing Date: 10 February 1989 (10.02.89)

(31) Priority Application Number: 154,730

(32) Priority Date: 11 February 1988 (11.02.88)

(33) Priority Country: US

(71) Applicant: BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM [US/US]; 201 West Seventh Street, Austin, TX 78701 (US).

(72) Inventors: OWEN-SCHAUB, Laurie, B. ; 4050 Drummond St., Houston, TX 77025 (US). GRIMM, Elizabeth, A. ; 2324 Bolsover, Houston, TX 77005 (US).

(74) Agent: PATTERSON, Melinda, L.; Arnold, White & Durkee, P.O. Box 4433, Houston, TX 77210 (US).

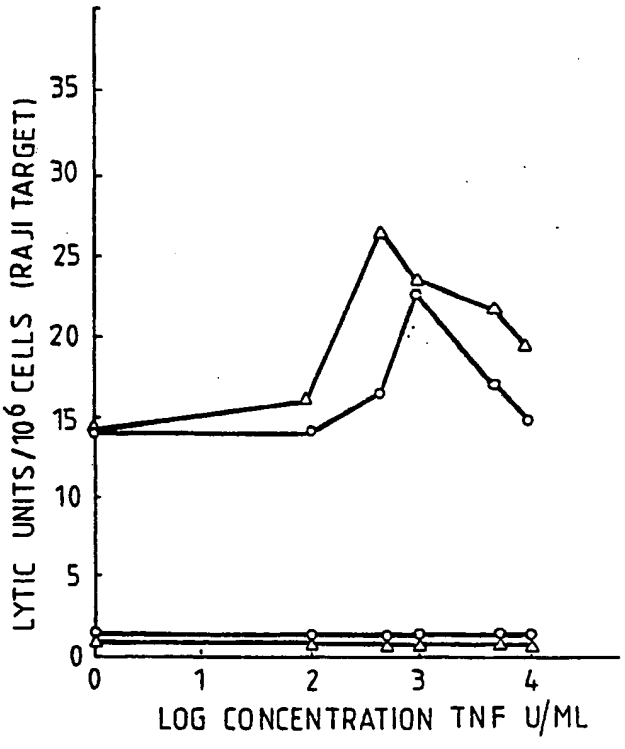
(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BG, BJ (OAPI patent), BR, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CM (OAPI patent), DE, DE (European patent), DK, FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL, NL (European patent), NO, RO, SD, SE, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: IMPROVED METHOD FOR TREATMENT OF CANCER WITH ACTIVATED ONCOLYTIC LEUKOCYTES

(57) Abstract

Tumor necrosis factor alpha (TNF-alpha) and tumor necrosis factor beta have now been shown to synergistically enhance the oncolytic function of human interleukin-2 (IL-2) activated lymphocytes (LAK). The presence of TNF-alpha or beta during activation consistently augments the LAK cytotoxic function two to four fold above that observed solely with IL-2. Furthermore, IL-2/TNF generated LAK, cultured with a ten-fold lower IL-2 concentration, demonstrate cytotoxic function equivalent to that observed with a ten-fold greater IL-2 alone stimulation, culture of lymphocytes with TNF alone is not sufficient for the generation of detectable LAK function. This novel synergy can be exploited under conditions in which the concentration of IL-2 would otherwise be limiting.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	ML	Mali
AU	Australia	GA	Gabon	MR	Mauritania
BB	Barbados	GB	United Kingdom	MW	Malawi
BE	Belgium	HU	Hungary	NL	Netherlands
BG	Bulgaria	IT	Italy	NO	Norway
BJ	Benin	JP	Japan	RO	Romania
BR	Brazil	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SN	Senegal
CH	Switzerland	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
DE	Germany, Federal Republic of	LU	Luxembourg	TG	Togo
DK	Denmark	MC	Monaco	US	United States of America
FI	Finland	MG	Madagascar		

IMPROVED METHOD FOR TREATMENT OF CANCER WITH ACTIVATED ONCOLYTIC LEUKOCYTES

The present invention relates to an improved method for generation of activated oncolytic leukocytes useful in the treatment of cancer. More particularly, the invention relates to the discovery that tumor necrosis factor (TNF) is synergistic with interleukin-2 (IL-2) in the generation of human lymphokine activated killer (LAK) cell cytotoxicity.

For decades, many skilled medical scientists have sought to develop an effective immunotherapy for the treatment of cancer. Although various approaches to achieving this goal have been tried, most have met with limited success. For example, in the 1970s, considerable attention was focused on efforts to induce tumor regression by general stimulation of the immune response with nonspecific immunostimulators such as Bacillus Calmette Guerin (BCG), Corynebacterium Parvum or Levamisole. Unfortunately, these experiments were generally unsuccessful.

In the 1980s, research efforts shifted from the infusion of exogenous nonspecific immunostimulatory agents

to administration of host proteins believed to play a specific role in inducing certain aspects of the immune response. These proteins, termed cytokines, are produced by cells of the host lymphoid or reticuloendothelial systems. Fortunately, many are now available as highly purified proteins produced in microorganisms as a result of recombinant DNA technology. Numerous cytokines, including various interferons, tumor necrosis factors, and various interleukins have been investigated as potential antitumor agents. Although results of certain of these studies appeared more promising than those described above, numerous problems were encountered. For example, one of the most effective agents, interleukin-2 (IL-2), was shown to have unacceptable toxicity at doses required for optimal chemotherapeutic effect. Therefore, alternative approaches were sought.

Some of the most promising results to date have been obtained with adoptive immunotherapy with the patient's own leukocytes. It has recently been shown that culture of peripheral blood lymphocytes with IL-2 elicits a dose dependent generation of potent oncolytic effector cells (References 1,2,3, full citation given in reference table below) designated lymphokine activated killer cells (LAK cells). More recently, Rosenberg and co-workers have shown that administration of an effective amount of interleukin-2 and interleukin-2-activated LAK cells can cause regression of a variety of cancers, including malignant melanoma, renal cell carcinoma, and colon cancer. These studies have resulted in U.S. Patent 4,690,915, incorporated herein by reference. Unfortunately, even in these studies, significant toxicity was observed as a result of the treatment. In addition, extremely high numbers of cells were required for infusion. Therefore, a method for optimal activation of the oncolytic effector cells was desired. Fortunately,

the present inventors have now discovered a method allowing surprisingly enhanced activation of oncolytic leukocytes.

It is, therefore, a general object of the invention to provide a novel method for optimal generation of potent oncolytic leukocytes useful for the treatment of cancer. Accordingly, the present invention includes a method for preparing oncolytic leukocytes for administration to a patient having cancer to induce regression of said cancer comprising obtaining a preparation of predominately mononuclear leukocytes from an individual and culturing the leukocytes in a suitable medium comprising effective amounts of both tumor necrosis factor and interleukin-2 to produce activated oncolytic leukocytes.

In addition, the invention also provides a method for treating cancer comprising identifying an individual having cancer, obtaining a preparation of predominately mononuclear leukocytes from said individual, culturing the leukocytes in a suitable culture medium containing effective amounts of both tumor necrosis factor and interleukin-2 to produce activated oncolytic leukocytes, and administering the oncolytic leukocytes to said individual to induce regression of said cancer. In further embodiments, the leukocytes will be administered together with IL-2 or with a composition comprising tumor necrosis factor and interleukin-2.

With the information produced by the present disclosure, it is believed that the respective concentrations of tumor necrosis factor and interleukin-2 that effectively produce an enhanced oncolytic leukocyte activation may be readily determined by those of skill in the art. However, in general it is preferred that the

amount of tumor necrosis factor in the culture medium range from about 2 ng/ml to about 100 ng/ml and that the amount of interleukin-2 range from about 1 ng/ml to about 100 ng/ml. Even more preferred is a tissue culture medium comprising from about 10-25 ng/ml of tumor necrosis factor and from about 3-50 ng/ml of interleukin-2. Furthermore, it will be appreciated that it is likely that any suitably purified source of interleukin-2 or tumor necrosis factor may be used to produce the synergistic activation. However, for practical reasons, it is preferred that recombinant interleukin-2 and tumor necrosis factor be used since purified preparations of these molecules are commercially available. Therefore, in a further embodiment, the tumor necrosis factor is defined as recombinant tumor necrosis factor. In an additional embodiment, the interleukin-2 is further defined as recombinant interleukin-2. In yet further embodiments, the tumor necrosis factor is more specifically defined as tumor necrosis factor alpha or tumor necrosis factor beta.

Finally, it will be appreciated that a method for treatment of cancer by administration of autologous interleukin-2 activated leukocytes together with interleukin-2 to induce regression of the cancer has been described previously. The present invention provides an improvement in this method comprising activating said leukocytes with a combination of interleukin-2 and tumor necrosis factor. As described previously, various amounts and types of interleukin-2 and tumor necrosis factor may be used.

Fig. 1 -- Stimulation of PBLs in serum-free media with: ▲, 10 units/ml IL-2 and TNF-alpha at the indicated concentration; △, TNF-alpha alone at the indicated concentration; ●, 10 units/ml IL-2 and TNF-beta at the indicated concentration; ○, TNF-beta alone at the indicated

concentration. After five days of activation, the PBLs were washed and tested in a ^{51}Cr release assay against the Raji target. These results indicate that either TNF-alpha or TNF-beta can synergize with IL-2 in the generation of LAK cytotoxicity.

Therefore, in accordance with the present invention, there is provided an improved method for optimal generation of activated oncolytic leukocytes, also referred to as lymphokine activated killer cells (LAK). The present inventors have made the surprising discovery that tumor necrosis factor (TNF), a polypeptide product of macrophages and lymphocytes initially recognized for its direct antitumor properties, synergistically enhances IL-2-mediated LAK activation. More specifically, the inventors have shown that the presence of TNF-alpha or tumor necrosis factor-beta during LAK activation consistently augments the LAK cytotoxic function. This discovery is likely to prove extremely useful in the production of such cells for adoptive immunotherapy of cancer.

In general, the methods used by the present inventors to generate the LAK activity parallel those described in U.S. Patent 4,690,915 with the striking and surprising difference that addition of TNF to the culture medium produces a synergistic enhancement of LAK activity.

To produce the synergistically activated oncolytic leukocytes of the present invention, one first obtains a fraction of leukocytes from an individual by leukophoresis or other known methods. Peripheral blood cells are preferred; however, with some patients it may prove desirable to obtain the leukocytes from other sources such as the spleen, lymph node, or tumor site.

Typically, particularly when peripheral blood lymphocytes are used, it is important to obtain a mononuclear fraction. This is usually achieved by fractionating the cells over a density gradient, for example, a Percol or Ficoll Hypaque gradient. The mononuclear cell fraction thus obtained may optionally be further purified. For example, the cell fraction may be depleted of monocytes or macrophages by plastic adherence; B cells may be removed by chromatography over nylon wool columns. Of course, other known methods for removing monocytes, macrophages, and B cells could also be used.

The isolated mononuclear cells are then be placed into culture in a suitable medium. The present inventors have shown that ideal activation is achieved with AIM V medium in the absence of serum and endotoxin. Of course, other suitable culture media, for example RPMI with human albumin, or human serum, may also be used. However, it is generally preferred that no serum or endotoxin be present in the medium.

Of course, it is important that the cells be stimulated with both TNF and IL-2. Although a number of suitable preparations of these agents may be used, it is preferable to use recombinant TNF and IL-2 since pure preparations of these proteins are commercially available and can be easily incorporated into the culture medium without the need for further purification. The lymphocytes are cultured with the synergistic stimulatory medium for approximately three to five days, preferably at a concentration of about 1×10^6 cells per ml.

After the culture period, the activated cells may then be transfused into a cancer patient to induce regression of the cancer.

The following examples describe actual synergistic activation of the LAK cells, assay of these cells in model systems, and how such cells could be used to induce tumor repression in vivo. These examples are intended to illustrate certain aspects of the present invention and should not be construed as limiting the claims thereof.

Peripheral blood cells obtained from normal volunteers by leukopheresis were fractionated on Histopaque (Sigma Chemical Co., St. Louis, MO) to produce a predominately mononuclear cell fraction. The resultant cells were adherence depleted on plastic two times and subsequently nylon-wool purified. Macrophage contamination of the PBL preparations was assessed by flow cytometric immunofluorescence using the Leu M3 antibody (Beckton Dickinson). The PBL used for these studies contained less than 1% Leu M3 positive cells.

The purified cells were then generally cultured at 1×6^6 PBL/ml of serum free AIM V media (Gibco Life Sciences, Grand Island, NY) containing L-glutamine, penicillin G (50 units/ml), streptomycin (50 ug/ml), and gentamycin (10 ug/ml) for four to five days at 37°C., 5% CO_2 with recombinant human IL-2 (rIL-2) (Cetus Corporation, Emeryville, CA), having a specific activity of 3×10^6 units/mg protein (8×10^6 Biological Response Modifier Program units/mg) and human recombinant TNF-alpha or human recombinant TNF-beta, obtained from Genentech, Inc., San Francisco, CA. The specific activity of the recombinant TNF-alpha (rTNF-alpha) used was 5×10^7 units/mg protein. The specific activity of the rTNF-beta was 1.2×10^8 units/mg protein. These TNF units are Genentech units. These reagents were generally added at the beginning of the culture period. However, presence of both IL-2 and TNF during the entire culture period may not be required. Preliminary experiments indicate that

addition of rTNF to rIL-2 activated PBL populations four to eight hours prior to the cytotoxicity assay is sufficient to produce enhanced oncolytic function.

Due to the difficulties inherent in testing new drugs and chemotherapeutic protocols in actual cancer patients, an in vitro assay was used to quantitate LAK activity. This assay, based on the well documented ability of activated LAK cells to kill fresh tumor cells and certain tumor cell lines, is generally accepted as an accurate assay of LAK activity in human systems. With this assay, cell suspensions from tumor and control tissues are utilized as targets in a four hour ^{51}Cr release assay to test for LAK cytotoxicity. Target cell lysis is detected by measuring release of the ^{51}Cr from the labelled target cell into the culture medium.

The assay was generally performed as follows. Target cells were labeled with 400 uCi of $Na^{51}CrO_4$ (Amersham Corp., Arlington Heights, IL) for 120 min. in 0.5 cm^3 of media. The target cells were washed four times with media and added in amounts of 5 x 10^3 cells/well to varying increments of the effector lymphocytes in 96 well round-bottom microtiter plates (Corning Glass Works, Corning, NY). The plates were centrifuged, incubated for four hours at 37°C., in an atmosphere of 5% CO_2. The cytotoxicity assay supernatants were harvested by means of the Skatron-Titertek System (Skatron Inc., Sterling, VA) and counted in a gamma counter. As controls, maximum isotope release was effected by incubation of the targets with 0.1 N HCl. Spontaneous release was assessed by incubation of targets with media alone. Fresh tumor targets were found to express between 10 and 30% spontaneous release during the four hour assay. The percentage of specific lysis was calculated by the formula

[(experimental cpm -- spontaneous cpm)/(maximal cpm -- spontaneous cpm)] x 100%.

For the calculation of lytic units, percentage specific lysis values from at least four effector:target ratios were used. The use of multiple effector:target ratios allows approximation of the sigmoidal killing curve. A line of best fit for the linear region of the killing curve was calculated and the number of effectors required to cause a given percentage target lysis extrapolated (usually 30%). This number is designated as 1 LU. Results are reported as LU/10^6 effectors.

Although the IL-2/TNF activated LAK consistently demonstrate augmented cytotoxicity against fresh tumor targets, a human cell line (Raji, American Type Culture Collection No. CCL 86) derived from a patient with Burkitt's lymphoma was chosen to assay cytotoxic potential of the lymphokine-activated killer cells in certain of the studies described below because of its reported resistance to direct TNF-alpha toxicity (4).

Initial studies were performed in order to determine the optimal concentration of IL-2 for LAK activation. In these studies, significant LAK activation was observed with about 100 u/ml of IL-2 (33 ng/ml); significant but suboptimal lysis of both Raji and fresh tumor targets was observed at IL-2 concentrations as low as 10 u/ml (3.3 ng/ml).

In other experiments, a range of TNF concentrations were tested for potential synergy. When human PBL were cultured in the presence of TNF with 10 u/ml of IL-2 (3.3 ng/ml), significant augmentation of LAK function occurred (Fig. 1). Augmentation was apparent over a range of TNF concentrations, with most PBL donors showing maximal

enhancement between 500 and 1000 units TNF/ml (10-20 ng/ml). This augmentation occurred with both TNF alpha and beta. Under the conditions used, in the absence of IL-2, neither TNF alpha or beta (tested at concentrations ranging from 10 to 10,000 units/ml) were able to generate LAK.

As stated above, the synergistically activated oncolytic leukocytes were assayed for their ability to kill both fresh tumor targets and a cultured tumor cell line. The following experiment describes efficacy of the synergistically activated leukocytes in killing fresh tumor targets.

Normal peripheral blood leukocytes were cultured for five days at 37°C., 5% CO_2, in serum-free medium at a concentration of 1 x 10^6 cells per ml. The IL-2 concentration used for these experiments was 10 u/ml (3.3 ng/ml, 0.2 nM). Where indicated, TNF-alpha was present at 500 u/ml (10 ng/ml 0.6 nM) during the activation period. After activation, the cells were harvested, washed and used as effectors in a ^{51}Cr release assay as described above. Fresh human tumor targets of various histological types which had been enzymatically disaggregated and cryopreserved as described previously (2) were used as targets. The lytic potency of the effectors derived from such activation can be compared using the lytic units (LU). The data are reported as the number of LU generated/10^6 cells.

As shown by Table I, synergistic activation was apparent using fresh tumor targets of several types. None of the targets tested demonstrated an increased spontaneous ^{51}Cr release in the presence of TNF-alpha or the combination of IL-2/TNF-alpha. Furthermore, addition of TNF-alpha into the ^{51}Cr release assay with the IL-2-

activated PBLs did not result in lytic augmentation. These results indicate that the synergistic lytic activity observed with IL-2 and TNF-alpha is not the result of direct target toxicity.

TABLE I

LYSABILITY OF FRESH HUMAN TUMOR TARGETS BY LAK ACTIVATED WITH IL-2 AND TNF-ALPHA

TUMOR TYPE	LU/10^6 EFFECTORS - TNF	+ TNF	FOLD INCREASE WITH TNF
Squamous cell lung carcinoma	64.4	136.2	2.1
"	0.4	10.8	30.8
"	20.6	48.0	2.3
Lung adenocarcinoma	39.6	62.7	1.6
Osteosarcoma	0.7	3.4	4.9
Osteosarcoma	11.5	35.5	3.1
Soft Tissue Sarcoma	5.4	12.8	2.4

Because many tumors are known to be sensitive to the direct toxic effects of TNF-alpha, the remainder of the studies were carried out with the TNF-alpha-resistant Raji cell line (4) for consistent analysis of the immunomodulatory effects of TNF-alpha. In these studies, varying doses of IL-2 in combination with 500 units/ml (10 ng/ml, 0.6 nM) TNF-alpha were examined for their effect on LAK induction. The synergistic effect of IL-2/TNF-alpha was observed over a wide range of IL-2 concentrations (Table II).

Both the magnitude of the cytotoxic enhancement and the IL-2 concentration that produced maximal synergy with

TNF-alpha were somewhat dependent upon the PBL donor. In the majority of donors (ten of twelve), represented by PBL A, synergistic activation was most notable when lower doses of IL-2 were used. However, two of twelve donors, represented by PBL B, demonstrated synergistic enhancement of cytotoxicity by TNF-alpha when IL-2 concentrations up to 1000 units/ml (330 ng/ml, 20 nM) were tested.

TABLE II

EFFECT OF IL-2 DOSE ON TNF-ALPHA SYNERGY

IL-2 CONCENTRATION	TNF ADDITION	LYTIC UNITS/10^6 EFFECTORS (RAJI TARGET) PBL A	PBL B
0	-	<0.1	<0.1
0	+	<0.1	<0.1
10	-	14.0	8.2
10	+	39.2	21.3
100	-	23.6	22.0
100	+	44.2	33.4
500	-	49.4	28.1
500	+	47.2	36.3
1000	-	65.4	38.4
1000	+	66.7	58.0

PBLs were activated for five days in serum-free media at a concentration of 1 x 10^6 cells/ml. TNF-alpha, where indicated, was present at 500 units/ml during the entire activation period.

Lytic units against the Raji target were calculated as described in "Materials and Methods."

The augmentation observed was not additive as PBL cultured with TNF-alpha in the absence of IL-2 remained ineffective against the NK-resistant Raji target. It is particularly significant that the synergistic enhancement

of LAK function observed with TNF-alpha and IL-2 consistently enabled production of optimal oncolytic activity (equivalent to or exceeding that using 100 u/ml IL-2 stimulation alone) at a 10-fold lower IL-2 concentration. These results suggest that the use of low dose IL-2, in combination with TNF-alpha, can reduce the IL-2 concentration required for optimal LAK activation.

In order to examine the specificity of the cytotoxic augmentation, PBL were activated with IL-2/TNF-alpha in the presence of a polyclonal antisera against human TNF. One ul of this antisera effectively neutralized 100 units TNF-alpha. As shown by Table III, LAK cytotoxicity was reduced to IL-2 control levels when anti-TNF was present during the four day activation period. In parallel experiments using normal rabbit serum, no abrogation of the cytotoxic enhancement was observed (data not shown). Therefore, these experiments indicate that the synergy is the specific result of TNF addition.

TABLE III

EFFECT OF TNF-SPECIFIC ANTISERA ON EXOGENOUS TNF ADDITION AND IL-2 ALONE-STIMULATED PBLs*

CULTURE CONDITIONS	ANTI-TNF	LYTIC UNITS/10^6 EFFECTORS RAJI TARGETS
IL-2	-	4.7
IL-2	+[a]	5.2
IL-2 + TNF	-	11.4
IL-2 + TNF	+	5.1
TNF	-	<0.1
Media	+	<0.1

*PBLs were cultured for five days in serum-free media containing IL-2 at 10 units/ml with or without TNF-alpha at 500 units/ml.

[a]Polyclonal rabbit anti-TNF antisera was present during the activation period at 500 neutralizing units/ml.

As has been pointed out above, LAK cells are characterized as killing both selected cell lines and fresh tumor targets. Although a TNF resistant line was purposely chosen as a target to ensure that the killing observed was due to the LAK cells, not to TNF that might have been carried over from the culture medium, the following experiment was performed in order to more conclusively demonstrate that the enhanced cytotoxicity observed when lymphocytes were cultured with TNF was due to enhanced activation of the lymphocytes, not to toxicity of TNF.

PBL were activated in serum-free media as described above. IL-2 was present at 0, 10, or 100 units IL-2/ml (0, 0.22 nM, 2.22 nM, respectively). After four days culture, viable cells were harvested, washed, and tested for cytotoxicity against the Raji target (2). TNF-alpha (0.6 nM) was present only during the four hour killing assay. Lytic units for each effector group were calculated as described. Spontaneous release of the Raji target in media alone = 754 $\pm$ 58; with TNF-alpha = 724 $\pm$ 31.

The results of this experiment, shown below in Table IV, demonstrated that inclusion of TNF-alpha in the four hour cytotoxicity assay did not alter the spontaneous release of the Raji target, nor did it significantly augment tumor lysis by LAK. Similarly, no evidence of cytotoxicity was observed with the fresh tumor cells shown in Table I. These results confirm that the synergistic effect(s) are manifest during the IL-2 driven generation

of LAK and occur independently of tumor target sensitivity to TNF-alpha.

TABLE IV

TNF-ALPHA INCUBATION WITH IL-2 STIMULATED PBL IS REQUIRED FOR AUGMENTED CYTOTOXICITY

IL-2 CONCENTRATION	TNF ADDITION TO ASSAY	LU/106 RAJI
-	-	< 0.1
-	+	< 0.1
10	-	9.7
10	+	9.7
100	-	12.6
100	+	13.0

The following experiments were then performed in order to investigate the mechanism of IL-2/TNF-alpha synergy. PBLs which had been activated for 5 days with IL-2 or IL-2/TNF- alpha were placed in flat-bottomed 96-well plates (Corning Glass Works) at 5 x 10^4 cells per well in quadruplicate in volumes of 0.150 ml. Each well received 1 uCi [^{3}H]thymidine (6.7 Ci/mmol specific activity; New England Nuclear) in a 0.050-ml volume. Cells were incubated for four hours at 37`C. and the plates harvested using a Ph.D. cell harvester (Cambridge Technology, Inc., Cambridge, MA). Individual filter discs were countered on a scintillation counter and the data expressed as the mean cpm $\pm$ SEM.

As shown in Table V, no detectable increase in [^{3}H]thymidine incorporation was observed when five-day PBLs cultured with IL-2/TNF-alpha were compared to PBLs cultured in IL-2 alone. Furthermore, cell recoveries from

the cultures containing IL-2/TNF-alpha were not increased compared to those from cultures containing IL-2 alone. Despite the fact that no augmentation of cellular proliferation was detectable, stimulation with IL-2/TNF-alpha resulted in an increased percentage of IL-2 receptor-bearing cells identified by the Tac epitope (5). These results suggest that augmented LAK effector function is not the sole result of increased proliferation.

TABLE V

TNF-ALPHA CULTURE INCREASES IL-2 RECEPTOR EXPRESSION BUT NOT PROLIFERATION IN PBLs

Culture Condition[a]	[^{3}H]thymidine Incorporation (cpm ± SD)[b]	Cell Recovery[c]	Percentage of IL-2 Receptor Expression[d]
IL-2	9298 ± 778	9.5×10^6	47.8
IL-2 + TNF	7746 ± 398	6.2×10^6	67.5
Media alone	415 ± 37	2.9×10^6	2.3

[a]PBLs cultured in serum-free media with no addition of cytokine, 10 units/ml IL-2, or 10 units IL-2 and TNF-alpha at 500 units/ml, for five days.

[b]Quadruplicate wells, 5×10^4 cells/well, 4 hour pulse as described in "Materials and Methods."

[c]Total cells recovery from a flask which had originally been seeded with 1.5×10^7 cells at 1×10^6 cells/ml.

[d]Immunofluorescence. Cells were indirectly stained with 10 ug/10^6 cells 7G7/B6 antibody and a 1:20 dilution of fluorescein isothiocyanate-labeled goat anti-mouse IgG antibody (Ortho Diagnostic Systems) to examine IL-2 receptor expression. The cells were then analyzed with a FACScan flow cytometer (Becton Dickinson). The data are presented as the percentage of cells positive after subtraction for nonspecific staining with an irrelevant monoclonal antibody and the goat anti-mouse secondary.

Therefore, the present inventors have clearly shown that IL-2 and TNF (both alpha and beta) synergize to produce a surprisingly enhanced activation of tumor reactive lymphocytes. Although the mechanism underlying this enhanced LAK effector function has not been fully elucidated, it may involve effector cell hyper-activation (increased rate of target cell killing and recycling) or

increased numbers of cytolytic cells via activation of diverse lymphocyte sub-populations. Initial experiments performed by these inventors indicate that the culture of PBL with IL-2 and TNF-alpha increases the rate of target cell killing. Whethe this is the result of effector cell enrichment is presently being addressed. Regardless of the mechanism involved, his IL-2/TNF synergy represents an important and previously unrecognized component of nonspecific immune amplification of lymphocyte mediated cytotoxicity which is likely to have wide-reaching therapeutic applicability.

EXAMPLE II

The following example describes how activated oncolytic leukocytes prepared according to the protocol of the present invention may be used to treat cancer patients.

Leukocytes are activated essentially as described above except that the culture protocol must be scaled up to accommodate the higher number of cells required for infusion into a patient. It is presently believed that treatment may require from about 10^6 to 10^{10} cells per patient. Therefore, especially when large numbers of cells to be infused, leukocytes donors will often be required to undergo repeated leukopheresis. A continuous flow separator, such as the Fenwal CS3000, (Fenwal Laboratories, Deerfield, IL) or Spectra (Cobe Laboratories, Lakewood, CO), may be used to effect the cell separation. Using this apparatus, in general, 5×10^9 to about 5×10^{10} mononuclear cells may be collected in each procedure. Ten to 12 liters of whole blood may be processed in approximately four hours to achieve this cell yield at a flow rate of about 60-70 ml/min. Suitable anti-coagulants such as acid citrate dextrose or heparin

should be used to prevent clotting. Mononuclear cells may then be separated using Histopaque (Sigma, St. Louis, MO) or similar density gradients and introduced into culture essentially as described above. However, since large numbers of cells are to be cultured, it may be preferable to use large culture vessels, for example, 175 cm^2 culture flasks (Corning) or bags (PL 732, Fenwal Laboratories) with a culture period of about three to about five days. The remainder of the culture conditions will preferably be similar to those described above, for example, about 10^5 to about 10^7 cells/ml of serum free medium containing 1-100 ng/ml of IL-2 and 2-100 ng/ml TNF-alpha.

After activation, the cells are harvested by centrifugation, washed in a suitable buffer; (for example, HBSS without calcium, magnesium or phenol red), and resuspended in infusion medium. A suitable infusion medium will comprise about 200 ml of 0.9 sodium chloride containing 5% normal human serum albumin and up to about 75,000 units of recombinant IL-2. The final cell suspension should generally be filtered to remove clumps, for example, through sterile Nitex (110 mesh) (Lawsh Instrument Company, Rockville, MD), and then transferred to a suitable transfer pack (for example, the Fenwal 4R2024), if activated in culture flasks, or infused directly if using Fenwal PL 732 bags.

Of course, prior to infusion of the cells and/or the IL-2, it is advisable to ensure that no bacterial contamination or endotoxin is present. Therefore, the cells may be assayed by standard techniques, for example, Gram stain, to ensure that no bacteria are present. The culture medium and/or IL-2 can be assayed for endotoxin, for example, by the Limulus amebocyte assay.

The activated oncolytic leukocytes will be infused either directly into the blood stream or into the tumor site. For example, the cells may be administered intravenously through a central venous catheter into a large peripheral vein, by direct infusion into the hepatic artery via a percutaneous catheter, or by intraoperative injection into the tumor site (6), or similar methods known to those of skill in the art. At the present time, it is believed that an optimal infusion protocol will comprise an initial infusion of approximately 1-5% of the total number of cells to be infused followed several minutes later by infusion of additional cells over approximately 20-30 minutes. The infusion bag should be mixed periodically during the process to prevent clumping of cells. Recombinant IL-2 may be similarly administered. For example, the IL-2 may be diluted in a suitable buffer, for example, normal saline with 5% human serum albumin, and infused intravenously over a 15 min. period. Repeated infusions of IL-2, for example, every eight hours, may be provided as desired and as taught by Rosenberg, et al., U.S. Patent 4,690,915.

Finally, it will be appreciated by those skilled in the art that IL-2 can be quite toxic for some patients. Therefore, any patient receiving IL-2 should be monitored carefully for such toxicity and treated appropriately. However, in that the present invention allows optimal activation of lymphokine activated oncolytic cells in vitro, it may be that administration of the lymphokine activated cells alone will produce sufficient antitumor response to obviate the necessity for administration of IL-2. This hypothesis has not yet been tested, but the present inventors believe that it is feasible. Alternatively, exogenous administration of low dose IL-2 or IL-2 in combination with TNF-alpha may be administered.

* * *

The foregoing description of the invention has been directed to a particular preferred embodiment in accordance with the requirements of the patent statutes and for purposes of explanation and illustration. It will be apparent, however, to those skilled in this art that many modifications and changes in both apparatus and method may be made without departing from the scope and spirit of the invention. For example, although certain embodiments of the invention relate to administration of autologous lymphocytes, murine studies indicate allogenic cells may be equally or more effective in some patients. (7). In addition, direct organ infusion (6) may be more effective than systemic administration of the activated cells. Furthermore, the activated cells may be further expanded in tissue culture prior to their administration. Also, it should be pointed out that the present invention is not expressly limited to LAK cells. For example, subsequent to the conception of the present invention, the startling report was made that IL-2 and TNF-alpha can enhance activation of natural killer cells, lymphocytes generally believed to represent a different population of cells than LAK cells (8).

It will be further apparent that the invention may also be utilized with suitable modifications within the state of the art; for example, the activated cells could be utilized together with other chemotherapeutic regimens. Examples of such known techniques include tumor cell-specific monoclonal antibodies and/or tumor reduction protocols, for example, chemotherapy, surgery, irradiation, and the like. These, and other modifications of the invention will be apparent to those skilled in this art. It is the Applicants' intention in the following claims to cover all such equivalent modifications and

variations which fall within the true spirit and scope of the invention.

REFERENCES

The following references may be useful in assisting understanding or practice of certain aspects of the present invention. Accordingly, each is expressly incorporated herein by reference.

1. E.A. Grimm, A. Mazumder, H.Z. Zhang, S.A. Rosenberg, J. Exp. Med., 155:1823-1841 (1982).

2. E.A. Grimm and S.A. Rosenberg, in Lymphokines, E. Pick, editor, Academic Press, Inc., New York, pp. 279-311 (1984).

3. E.A. Grimm, Biochimica et Biophysica Acta, 865:267-279 (1986).

4. S.C. Wright and B. Bonavida, J. Immunol., 138:1791-1798 (1987).

5. Rubin, et al., Hybridoma, 4:91-102 (1985).

6. Jacobs, et al., Cancer Research, 46:2101-2104 (1986).

7. Mule, et al., J. Immunol., 135:646-652 (1985).

8. Ostensen, et al., J. Immunol., 138:4185-4191 (1987).

CLAIMS:

1. A method for preparing oncolytic leukocytes for administration to a patient having cancer to induce regression of said cancer comprising:

obtaining a preparation of predominantly mononuclear leukocytes from an individual; and

culturing the leukocytes in a suitable culture medium comprising tumor necrosis factor and interleukin-2 to produce activated oncolytic leukocytes.

2. A method for treating cancer comprising:

(a) obtaining a preparation of predominantly mononuclear leukocytes from an individual having cancer;

(b) culturing the leukocytes in a suitable culture medium comprising tumor necrosis factor and interleukin-2 to produce activated oncolytic leukocytes; and

(c) administering the activated oncolytic leukocytes to said individual to induce regression of said cancer.

3. The method of claim 2 wherein the leukocytes are administered together with interleukin-2.

4. The method of claim 2 wherein the leukocytes are administered together with a composition comprising tumor necrosis factor and interleukin-2.

5. The method of claim 1 or 2 wherein the amount of tumor necrosis factor in the culture medium ranges from about 2 ng/ml to about 100 ng/ml and the amount of interleukin-2 ranges from about 1 ng/ml to about 100 ng/ml.

6. The method of claim 1 or 2 wherein the tumor necrosis factor is further defined as recombinant tumor necrosis factor.

7. The method of claim 1 or 2 wherein the tumor necrosis factor is further defined as tumor necrosis factor alpha.

8. The method of claim 1 or 2 wherein the tumor necrosis factor is further defined as tumor necrosis factor beta.

9. The method of claim 1 or 2 wherein interleukin-2 is further defined as recombinant interleukin-2.

10. In a method for treating cancer by administering to a patient suffering from cancer autologous interleukin-2 activated leukocytes together with interleukin-2 to induce regression of said cancer; the improvement which comprises:

activating said leukocytes with a combination of interleukin-2 and tumor necrosis factor.

11. The method of claim 10 wherein the leukocytes are activated by culture in a medium comprising about 2 ng/ml to about 100 ng/ml tumor necrosis factor and about 1 ng/ml to about 100 ng/ml interleukin-2.

12. The method of claim 10 wherein the tumor necrosis factor is further defined as recombinant tumor necrosis factor.

13. The method of claim 10 wherein the tumor necrosis factor is further defined as tumor necrosis factor alpha.

14. The method of claim 10 wherein the tumor necrosis factor is further defined as tumor necrosis factor beta.

15. The method of claim 10 wherein interleukin-2 is further defined as recombinant interleukin-2.

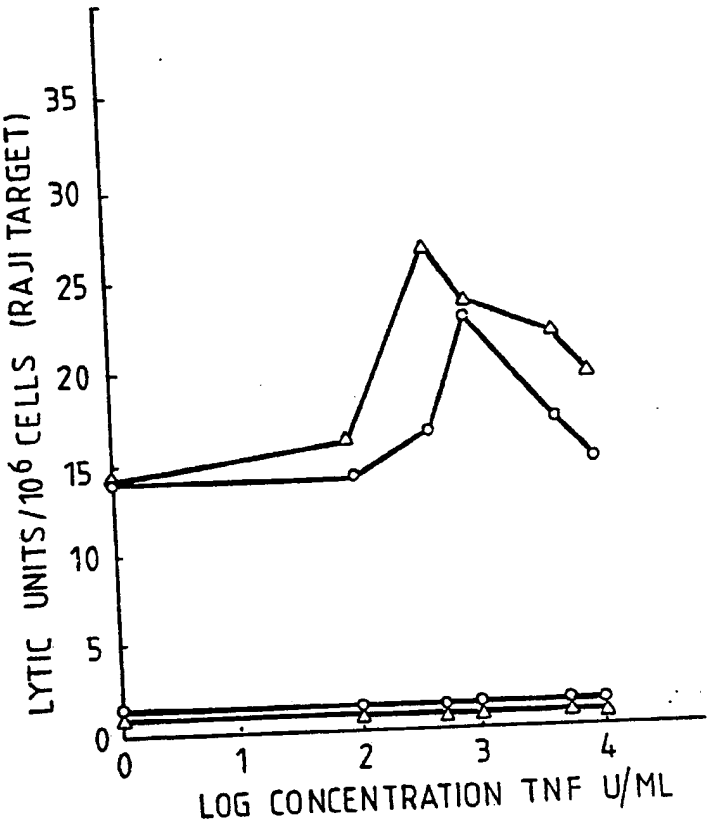

LYTIC UNITS/10^6 CELLS (RAJI TARGET)
35
30
25
20
15
10
5
0
0
1
2
3
4
LOG CONCENTRATION TNF U/ML

Fig. 1

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/00552

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: A 61 K 35/14; A 61 K 37/02; // C 12 N 5/00

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
IPC[4]	A 61 K; C 12 N; C 12 P

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched *

III. DOCUMENTS CONSIDERED TO BE RELEVANT *

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
Y	The New England Journal of Medicine, volume 313, no. 23, 1985, S.A. Rosenberg et al.: "Special Report. Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer", pages 1485-1492 see front-page, abstract; page 1486, column 2, "Lymphocyte harvest and culture" --	1,5-9
Y	The Journal of Immunology, volume 138, no. 12, 15 June 1987, The American Association of Immunologists, (US), M.E. Østensen et al.: "Tumor necrosis factor-α enhances cytolytic activity of human natural killer cells", pages 4185-4191 see front-page, abstract; page 4186, "Cell preparations" and "Culture conditions for generation of activated	1,5-9

./.

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
23rd May 1989	12 JUN 1989
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	P.C.G. VAN DER PUTTEN

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	killer cells", page 4187, figure 3; pages 4189-4190, "Discussion" cited in the application	
	--	
X,P	Cancer Research, volume 48, no. 4, 15 February 1988, L.B. Owen-Schaub et al.: "Synergy of tumor necrosis factor and interleukin 2 in the activation of human cytotoxic lymphocytes: effect of tumor necrosis factor α and interleukin 2 in the generation of human lymphokine-activated killer cell cytotoxicity", pages 788-792 see the whole article	1,5-9
	--	
X,P	Chemical Abstracts, volume 110, no. 9, 28 February 1989, (Columbus, Ohio, US), C. Kasai: "Experimental studies on recombinant cytokine for malignant bone tumors", see pages 479-480, abstract 73610y, & Gifu Daigaku Igakubu Kiyo 1988, 36(3), 426-56	1,5-9
	--	
A	Biolgocal Abstracts, volume 86, no. 4, 1988, (Philadelphia, PA., US), J. Gangi et al.: "Anti-tumor activity and production of cytotoxic factor by lymphokine-activated killer cells", see page AB-692, abstract 39922, & Okayama Igakkai Zasshi 99(11/12): 1403-1410, 1987	1,5-9
	--	
A	EP, A, 0248516 (CETUS CORP.) 9 December 1987 see the whole document	1,5-9

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers ....*.... because they relate to subject matter not required to be searched by this Authority, namely:

* 3-4, 10-15

See PCT-Rule 39.1 (iv): methods for treatment of the human or animal body by surgery or therapy as well as diagnostic methods.

2. [ ] Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers ....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 8900552 SA 26914

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 08/06/89
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0248516	09-12-87	AU-A- 7117687	15-10-87
		JP-A- 62242629	23-10-87